Primary Pulmonary Hypertension Between Inflammation and Cancer*

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We believe that the monoclonal cell expansion in primary pulmonary hypertension is the result of autonomous growth of stem cell-like endothelial cells, whereas the polyclonal proliferation in secondary pulmonary hypertension occurs as a response of endothelial cells to exogenous stimuli (like viral infection or high shear stress). In this context, we propose that different transcriptional and translational events govern the growth and expansion of monoclonal when compared with polyclonal pulmonary endothelial cells. The availability of antibodies directed against specific tyrosine kinase proteins involved in vasculogenesis/angiogenesis now permits the identification and localization of the components of such a misguided angiogenesis cell proliferation program in the pulmonary hypertensive vascular lesions. (CHEST 1998; 114:225S-230S)

In very broad terms, severe pulmonary vascular disease can histologically be classified by the cell type that contributes most to the pulmonary vascular remodeling. If chronic hypoxia is an important contributor to the pulmonary vascular remodeling, as in chronic obstructive lung disease or as in some forms of interstitial fibrosis, then vascular smooth muscle cell hypertrophy and proliferation characterize the vascular alterations and endothelial cell proliferation is absent. In contrast, in all forms of the plexogenic arteriopathy (including primary pulmonary hypertension [PPH], severe pulmonary hypertension associated with collagen vascular disease, portal hypertension, and HIV-associated severe pulmonary hypertension), endothelial cell proliferation is histologically apparent and can be impressively documented immunohistochemically using antibodies directed against factor VIII-related antigen or against the vascular endothelial growth factor (VEGF) receptor KDR.

Figure 1 provides a synopsis of the structural abnormalities that characterize PPH and shows the pruning of the pulmonary vascular tree and the oblitative lesions of the small precapillary vessels. One of the major unanswered questions in pulmonary vascular research is why conditions as diverse as viral infection, shear stress, portal hypertension, and drug use all basically generate the same pulmonary vascular lesions (Fig 2). There is now general agreement that a genetic predisposition is required for the development of severe, progressive, and likely irreversible, pulmonary vascular disease.

Figure 3 shows that the progressive vascular remodeling leads to a loss of vascular reactivity and increased pulmonary vascular shear stress that leads to further vascular remodeling. What is presently unclear is how exactly vasoconstriction, inflammation, and perhaps primary endothelial cell proliferative activity are linked and whether the genetic susceptibility codes for vasoconstriction or cell proliferation or both.

On the endothelial cell level, cell activation or injury somehow triggers a cell growth program, which results in the growth of phenotypically altered and dysfunctional cells.

PULMONARY HYPERTENSION AND INFLAMMATION

It has been generally accepted that mediators of inflammation can cause pulmonary vasoconstriction, but it is less clear whether inflammatory cells or mediators of inflammation also contribute to the pulmonary vascular remodeling and, therefore, participate in the development of chronic pulmonary hypertension. In the context of pulmonary hypertension, inflammation is neither rubor nor calor, but the presence and the activity of inflammatory cells (including macrophages, polymorphonuclear neutrophils, lymphocytes and mast cells, and inflammatory cell-derived growth factors and cytokines). It is of further interest that not only chemoattractants may play a role in the development of chronic pulmonary hypertension, but also, that chronic hypoxia—in addition to causing vasoconstriction—can also cause the release of cytokines and growth factors from "professional" inflammatory cells. Heath and Yacoub demonstrated the presence of large...
amounts of mast cells in the vicinity of pulmonary arterioles from patients with precapillary and post-capillary pulmonary hypertension. Mast cells can release tryptase, a potent angiogenesis factor, as well as leukotrienes and proteases. Recently, Tuder and coworkers and Cool et al demonstrated accumulations of macrophages, and T and B lymphocytes in the vicinity of pulmonary vessels from patients with PPH. More recently, our group localized the 5-lipoxygenase (5-LO) and the 5-LO-activating protein (FLAP) to endothelial cells of pulmonary arteries (characterized by media hypertrophy) and to plexiform lesions in lung sections from PPH patients. The 5-LO and FLAP expression was more intense in the small- and medium-sized pulmonary arteries in PPH than in comparable normal lung vessels. The macrophages that were found in clusters surrounding remodeled pulmonary arteries exhibited a strong expression for both 5-LO and FLAP. Presently, it is not clear whether the presence of these proteins that relate to inflammation correlate with increased leukotriene synthesis. Humbert et al found increased interleukin-1 and interleukin-6 serum levels in patients with severe PPH and this group also localized platelet-derived growth factor (PDGF) A messenger RNA (mRNA) to perivascular macrophages in lungs of patients with PPH and HIV-related pulmonary hypertension. Interestingly, PDGF can act synergistically with hypoxia to induce VEGF. Certainly, platelets and factors released by activated platelets can play a role in the pulmonary vascular remodeling.

THE PULMONARY HYPERTENSIVE ENDOTHELIAL CELL PHENOTYPE

The endothelial cells comprising the plexiform lesions are morphologically different from the flat monolayer endothelial cells of patent pulmonary arteries. The endothelial cells of the plexiform lesions form a conglomerate of large cells with large nuclei; early lesions can be observed as solid tumors in the area of bifurcations. The cells that make up the plexiform lesions show abnormal expression of endothelin, 5-LO and FLAP, of VEGF, and
a diminished expression of the prostacyclin synthase. The expression in plexiform lesions of VEGF mRNA is consistent with the concept of autocrine endothelial cell growth, since endothelial cells usually do not express VEGF—only the VEGF receptors. Whether in PPH there is a decrease in the activity of pulmonary vascular endothelial nitric oxide production is not known. The data regarding the expression of the endothelial cell nitric oxide synthase are presently controversial; both decrease of expression and increase in expression in plexiform lesion have been reported. A decrease in thrombomodulin expression in plexiform lesions may relate to the enhanced thrombogenicity of the endothelial cell surface in severe pulmonary hypertension (PH).

PULMONARY ENDOTHELIAL CELL GROWTH IN PPH IS MONOCLONAL

It is presently unclear whether PPH is one single homogenous disease or a spectrum of diseases that have in common the development of endothelial cell proliferation within the plexiform and concentric lesions. It is intuitive that an answer to this question is extremely important. The pathogenesis and the natural history of different disease forms—if there are indeed different disease forms—may be different in patients, for example, with dexfenfluramine-induced disease when compared with patients who have an atrial septum defect and pulmonary hypertension.

In searching for early plexiform lesions, we discovered vascular sprouts, which consist of an accumulation of large endothelial cells. Because of the tumor-like appearance of these vascular sprouts, we asked the questions whether these cell clusters were of monoclonal or polyclonal nature. The rationale for our approach is described briefly below. One of the two X chromosomes is randomly inactivated in each female cell. The inactivated chromosome always remains the same throughout subsequent cell divisions. Thus, in the adult woman, there is a mosaic of two cell types differing in that either the paternal or maternal X chromosome has been inactivated. A clonal cell population, because it arises from the same progenitor cell, will maintain the same pattern of X chromosome inactivation that was present in the cell of origin.

Early studies of clonality of human tissues were severely limited by the low heterozygosity for X chromosome markers. The introduction of methods to analyze restriction fragment length polymorphisms partially alleviated these problems. X chromosome inactivation of many genes is mediated, at least in part, by hypermethylation of base residues, including cytosine. The restriction endonuclease HpaII cleaves the sequence 5'CtGG3, but it is inhibited by methylation of the internal cytosine and the 5' cytosome base. Pretreatment of the DNA extracted from tissues with HpaII will generate restriction fragments from the active, but not the inactive, X chromosome. The development of polymerase chain reaction methodology opened the door for the application of restriction fragment length polymorphism analysis to archival tissue collections.

More recently, a polymorphic short tandem repeat was identified in the X-linked human antigen receptor gene (HUMARA). This sequence consisting of the trinucleotide (CAG) shows heterozygosity in 90% of the general population. Subsequently, it could be shown that methylation of HpaII and HhaI sites within the HUMARA amplification target directly correlates with X chromosome inactivation.

We used archival frozen tissue sections, extracted the DNA, and used the methylation-sensitive restriction endonuclease (HhaI). This treatment was followed by polymerase chain reaction amplification of a portion of the X-linked HUMARA. Using the HUMARA approach, we analyzed 22 plexiform lesions from patients with PPH and 20 lesions from patients with secondary pulmonary hypertension.
The results so far indicate strongly that most of theplexiform and concentric endothelial cell proliferation lesions in the PPH are monoclonal. In contrast,none of the examined comparable lesions from patients with secondary PH were monoclonal.  

Monoclonality implies that a certain cell population descends from one single cell. Although no information, (to our knowledge) is available on the pattern of X chromosome inactivation in the normal endothelium, our finding that in secondary plexiform lesions there is always a polyclonal proliferation of endothelial cells indicates that the endothelial cell patch is smaller than the sampled area. It also appears that different plexiform lesions in the same PPH lung exhibit monoclonal endothelial cell proliferation with different patterns of X chromosome inactivation in different lesions.

Monoclonality of endothelial cells in PPH is the first molecular marker at the tissue level to categorically distinguish PPH from secondary PH related to congenital heart malformations and PH associated with collagen vascular diseases. By extending the concept of monoclonality to PH associated with HIV, portal hypertension, and dexfenfluramine-induced PH, we can now verify whether PPH consists of a homogenous disease driven by the expansion of monoclonal endothelial cells.

**Vasculogenesis and Angiogenesis**

Blood vessels are constructed by two processes: vasculogenesis, whereby a primitive vascular network is established during embryogenesis from multipotential mesenchymal progenitor cells; and angiogenesis, in which preexisting vessels (both in the embryo and in the adult) send out capillary sprouts to produce new vessels. It is now clear that endothelial cells are essential in vasculogenesis and angiogenesis. Support cells are recruited to encase the endothelial tubes, providing maintenance and modulatory functions for the vessels. Such cells include pericytes for small capillaries and smooth muscle cells for larger vessels. The establishment and remodeling of blood vessels are controlled by paracrine signals, many of which are protein ligands that modulate the activity of transmembrane receptor tyrosine kinases. During vasculogenesis, mesoderm-derived angioblasts differentiate into endothelial cells that form the novel vessels, including the dorsal aorta.

VEGF is essential to the process of vasculogenesis. Disruption of the gene for the VEGF receptor flk-1 (KDR receptor in humans) interferes with the differentiation of endothelial cells leading to death of the embryos at day 8.5 to 9.5. Disruption of the other VEGF receptor flk-1 permits differentiation of endothelial cells, but interferes with a later stage of vasculogenesis resulting in thin-walled vessels of larger than normal diameter and death of the embryos on day 9.0. In addition to the two VEGF receptor/kinases, Tie 2 kinase is another endothelial cell-specific receptor tyrosine kinase; the ligands for this receptor kinase are angiopoietin-1 (ANG-1) and ANG-2. The Tie 2 receptor tyrosine kinase is primarily coupled to signal transduction, which elicits vessel maturation and maintenance. The other ligand for the Tie 2 receptor tyrosine kinase is ANG-1, with its functional role of recruiting and sustaining periendothelial support cells. It is of interest that ANG-2 is selectively expressed in the ovary, uterus, and placenta, the three tissues subject to physiologic angiogenesis. There is evidence that other growth factors are also critically involved in vasculogenesis. For example, transforming growth factor-β null mice die from a severe defect in yolk sac vasculogenesis thought to result from improper interactions between epithelial and mesenchymal cells, whereas PDGF-receptor-β and PDGF-B ligand null mice die perinatally from hemorrhage and have no pericytes in their kidney vasculature. Likewise, mice deficient for tissue factor die in utero at day 8.5 with vascular abnormalities that appear to be due to a defect in recruitment of smooth muscle cells. Absence of ANG-1 also results in vessels that do not properly recruit supporting cells to the vascular walls.

Since there is a link between HIV infection and development of severe plexogenic PH (apparently in genetically susceptible individuals), it is important to mention in this context that the HIV tat protein (known to induce endothelial cell growth) can be angiogenic and that angiogenesis induced by the tat protein is mediated by the flk-1 VEGF receptor and by induction by VEGF mRNA.

New data are also being reported regarding a role for nitric oxide in angiogenesis. Ziche et al. provided evidence that nitric oxide mediates angiogenesis, endothelial cell growth, and migration. A particularly exciting piece of information emerged recently from an article by Maltepe and coworkers, who showed that knockout mice defective for the aryl hydrocarbon-receptor nuclear translocator (ARNT) have defects in vasculogenesis very similar to those observed in mice deficient in VEGF or tissue factor. This observation is important because it provides a potential link between the aryl hydrocarbon receptor, which has been implicated in carcinogenesis, vasculogenesis, and abnormal angiogenesis.

Figure 4 shows a flow diagram that organizes the information (signal transduction) from the level of gene transcription to the level of endothelial cell...
Receptor Tyrosine Kinase Activation Drives Lung Endothelial Cell Proliferation

![Diagram showing receptor tyrosine kinase activation drives lung endothelial cell proliferation.]

Figure 4. This schematic integrates the elements of our “misguided angiogenesis” hypothesis. Depicted is a hierarchy of events leading from increased gene transcription to the formation of proliferative, plexiform lesions. HIF-1α is hypoxia-inducible factor 1α, HIF-1β is identical with the aryl hydrocarbon receptor nuclear translocator, EPAS-1 is an endothelial cell-specific hypoxia-inducible transcription factor.

Conclusion

Endothelial cell proliferation is the one important, distinguishing aspect of severe progressive PH, whereas endothelial cell proliferation does not play a significant role in PH secondary to chronic obstructive lung disease or interstitial lung diseases. A recent case report documented plexiform lesions in the lung of a young woman who took anorexigenics and died from PPH approximately 8 months after the onset of drug use. This case report is perhaps the most convincing recent illustration of the fact that endothelial cell proliferation in PPH is not an end-stage scar-tissue event. In our experience, endothelial cell proliferation is present in all cases of PPH. Three-dimensional reconstruction of the obliterated small pulmonary arteries shows how extensive the endothelial cell proliferative component in PPH really is.

We hypothesize that VEGF plays an important role in the pathogenesis of PPH and other forms of severe PH. We further hypothesize that plexiform and concentric intima fibrotic lesions are the result of a process that we tentatively call “misguided angiogenesis.” We believe that the monoclonal cell expansion in PPH is the result of autonomous growth of stem cell-like endothelial cells, whereas the polyclonal proliferation in secondary PH occurs as a response of endothelial cells to exogenous stimuli (like viral infection or high shear stress). In this context, we propose that different transcriptional and translational events govern the growth and expansion of monoclonal when compared with polyclonal pulmonary endothelial cells. The availability of antibodies directed against specific tyrosine kinase proteins involved in vasculogenesis/angiogenesis now permits the identification and localization of the components of such a misguided angiogenesis program in the pulmonary hypertensive vascular lesions.

References

15. Humbert M, Monti G, Brenot F, et al. Increased interleukin-1 and interleukin-6 serum concentrations in severe pri...
primary pulmonary hypertension. Am J Respir Crit Care Med 1995; 151:1628-31
22 Folkman J, D’Amore PA. Blood vessel formation: what is its molecular basis? Cell 1996; 87:1153-56
23 Hanahan D. Signaling vascular morphogenesis and maintenance. Science 1997; 277:48-50
32 Maltepe E, Schmide JV, Bauno D, et al. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature 1997; 386:403-06
35 Cool C, Voelkel NF, Tudor RM. Three-dimensional reconstruction of the plexiform lesions in pulmonary hypertension. Am J Respir Crit Care Med 1996; 153:A40